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Incorporation of ^{14}C-Labeled 2,4,6-Trinitrotoluene Metabolites into Different Soil Fractions after Anaerobic and Anaerobic−Aerobic Treatment of Soil/Molasses Mixtures

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Experiments were conducted to evaluate the level of incorporation of ^{14}C-labeled 2,4,6-trinitrotoluene (TNT) and metabolites into the organic soil matrix of anaerobic and sequential anaerobic−aerobic treated soil/molasses mixtures. After 9 weeks of anaerobic−aerobic incubation with an optimized experimental setup, we determined nearly 84% of the initially applied radioactivity immobilized in different soil fractions, whereas only 57% of the radioactivity was measured as immobilized in the soil organic matrix at the end of the anaerobic treatment (after 5 weeks). After alkaline hydrolyses of the solvent-extracted soil/molasses mixtures, small amounts of radioactivity were found in the humic acid and fulvic acid fraction, whereas the major part of radiolabel was found to be strongly bound to the humin fraction. In agreement with these findings, the amount of extractable radioactivity (water, methanol, and ethyl acetate extractions) decreased from 40% after the anaerobic phase to nearly 9% after the aerobic treatment phase. The transformation of TNT at the end of the experiments was above 95% and 97% after anaerobic and sequential anaerobic−aerobic treatment, respectively. We propose a two-step treatment process (anaerobic−aerobic bioremediation process) with some special procedures during the anaerobic and the aerobic treatment phases as the most promising method for effective, economic, and ecologically acceptable disposal of TNT from contaminated soils by means of immobilization (for example, humification) of this xenobiotic.

Introduction

The relevant authorities and remediation companies of many industrialized countries have been making many efforts to develop and establish efficient and reasonable techniques for the cleanup of soil sites that are contaminated with explosives. 2,4,6-Trinitrotoluene (TNT) was the most widely produced and applied explosive in World Wars I and II (*1*). The remediation of soils and groundwater contaminated with TNT is of particular concern, since this compound and its reduced metabolites (e.g., aminodinitrotoluenes and diaminonitrotoluenes) are toxic to a variety of biota and show a broad spectrum of toxicological behavior ranging from mutagenic to carcinogenic activity (*2*−*7*). Various soil remediation techniques such as incineration, soil washing, or biological soil treatment were applied in the past, but the (micro)biological degradation of TNT-contaminated soils is considered to be the most favorable technique as far as costs are concerned (*8*). A very promising strategy in this context is to boost the bioremediation of contaminated soil with cheap biomass products such as alfalfa, sawdust, chopped potato waste, apple pomace, cow and chicken manure, straw, or molasses in compost systems (*1, 9*−*12*), which can lead to transformations of TNT of more than 95% (*1, 8, 11, 12*) and is often accompanied by detoxification effects (*8, 13*). The balancing of the TNT degradation in soil often yielded a balance gap of metabolites. This gap has been suggested to be due to an irreversible binding of TNT metabolites to the soil organic matrix. To close this gap, the scope of the present work was to estimate the actual fate of ^{14}C-labeled TNT in molasses-supplemented soil bioreactors in more detail after an anaerobic and a sequential anaerobic−aerobic treatment process. The use of radiolabeled TNT in soil reactors allowed us to monitor and quantify all putative distribution pathways in soil separately (solvent extracts, gas phase, humic acid fraction, fulvic acid and polyphenol fraction, and humin fraction) and should therefore help to distinguish the relative share of the two major possibilities of the fate of xenobiotics presumed to function in soil, namely, mobilization (e.g., wash-out effects) and immobilization (e.g., fixation by irreversible binding to the organic soil matrix). A sequential anaerobic−aerobic treatment of soil/molasses mixtures was chosen in which the anoxic conditions should lead to microbially produced polar metabolites of TNT (e.g., reduced metabolites) and in which a subsequent incorporation of these more polar metabolites into the soil matrix occurs under oxic conditions. Rieger and Knackmuss (*14*) and Lenke et al. (*15*) also recommended an anaerobic/aerobic bioremediation process, which they tested in a technical scale volume of up to 18 m^3 of contaminated soil, but exact data of the fate of radiolabeled TNT metabolites in different soil fractions are missing. Additionally, there were some differences in the treatment procedures in the present study as compared with the studies of the authors mentioned above (*14, 15*). To our knowledge, the present study is the first to show data that demonstrate the incorporation of nearly 84% of the originally applied radioactivity into the organic soil matrix as a result of the applied cleanup technique (sequential anaerobic−aerobic bioremediation process). In addition, results of the extractable radioactivity and the concentrations of TNT and its metabolites in different extracts will be presented and discussed.

Materials and Methods

Experimental Setup. In the first experiment (experiment A), the evaluation of carbon transfer from the ^{14}C-labeled TNT was carried out in closed soil/molasses bioreactors (250 mL volume) as depicted in the process scheme in Figure 1. The soil bioreactors were closed with butyl rubber stoppers; the gas phases were flushed with helium for 5 min to remove air oxygen and were then allowed to become anoxic (closed

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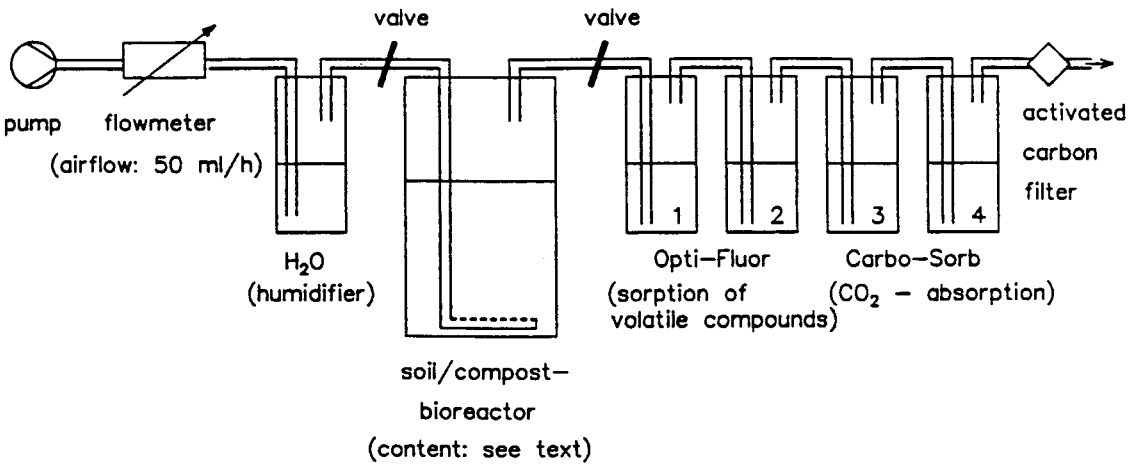

FIGURE 1. Process scheme of a soil/molasses bioreactor used in the entire experiment A and during the anaerobic treatment phase of experiment B.

valves in Figure 1). The exhaust gas that was produced in these anaerobically treated reactors was led through four sorption vessels (connected in series) and through an activated carbon filter by flushing with helium for 5 min at weekly intervals. Each sorption vessel was analyzed separately at the end of the experiment. In the first two gas-washing bottles containing 30 mL of Opti-Fluor (from Packard Instrument B.V. Chemical Operations, Groningen, The Netherlands), volatile organic compounds were adsorbed, followed by two additional vessels with 30 mL of Carbo-Sorb (also from Packard Instrument B.V. Chemical Operations) for the adsorption of radiolabeled carbon dioxide. After a 5-week anaerobic treatment, the bioreactors were aerated by opening the valves (see Figure 1) and by leading moistened air from the bottom of the reactors through the soil/molasses mixtures at a flow rate of 50 mL/h. This ventilation was carried out for an additional 4 weeks. All reactors were incubated at 35 °C in the dark. Three bioreactors (one spiked with [^{14}C]TNT, one spiked with normal TNT, and one unspiked control) were analyzed after the anaerobic treatment (after 5 weeks), whereas three further bioreactors (again one with [^{14}C]TNT, one with normal TNT, and one control) were analyzed after the additional aerobic treatment of 4 weeks (in sum 9 weeks).

In the second experiment (experiment B), a different bioreactor design was performed. While the soil/molasses mixtures were stacked in the bioreactors as loose piles during the anaerobic treatment in experiment A, the mixtures were strongly compressed under helium flush to remove air oxygen and to minimize space (e.g., reduction of pore size) for facilitating microbial metabolic activities by shortening of distances in experiment B. A mortar was used for soil compression. During the aerobic treatment, the soil/molasses mixtures were aerated with moistened air through the loose piles but were left unstirred in experiment A, whereas the compressed piles in experiment B were mixed thoroughly once a day but were not recompressed. These mixtures were incubated without rubber stoppers and were allowed to use the normal air oxygen. The loss of moisture content of the soil was corrected daily by spraying corresponding quantities of new tap water over the mixtures (to keep the moisture level constant). The absorption of radiolabeled volatile organic compounds and CO_2 was omitted in the aerobic treatment of experiment B because the yields of these compounds in the gas phase in experiment A (see Results section) were shown to be negligible.

Soil/Molasses Mixture Preparation. The soil material was collected from contaminated sites of a former ammunition plant, called Tanne, near Clausthal-Zellerfeld (Lower Saxony, Germany) and was passed through a sieve with 2 mm diameter openings. The soil contained ~32% clay and silt, ~63% sand, and ~5% gravel and had a pH of 6.9−7. The concentration of TNT of the contaminated soil was approximately 1180 mg/kg of dry soil (mean value of six parallel determinations). The methanolic extraction procedure for TNT determination was previously described by Breitung et al. (*16*). A total of 20 g of soil (dry weight) containing the above-mentioned contamination was mixed with 20 g of uncontaminated soil (dry weight) from the same TNT production plant to achieve an initial TNT concentration of 590 mg/kg of dry soil. Ten grams of molasses slivers (sugar content ~22.5 wt %; purchased from Südzucker AG, Warburg, Germany) was added to the soil as additional and stimulating carbon source for microbial activities to give an end concentration of soil and molasses of 80:20 wt % (50 g of mixtures [dry weight] per bioreactor). The water content of the mixtures was adjusted to 30 wt % at the beginning of the experiments. These soil/molasses mixtures were filled into the reactors (six in both experiments) and were contaminated (spiked) additionally with radiolabeled or nonradiolabeled TNT (exept the controls). In both experiments (A and B), two mixtures were externally spiked with [^{14}C]TNT at a concentration of 100 mg/kg of dry soil, and two mixtures were externally spiked with normal TNT (100 mg/kg of dry soil) to give an initial concentration of 690 mg of TNT/kg of dry soil. As controls in both experiments, two additional mixtures of uncontaminated and unspiked soil and molasses (80:20 wt %) were incubated. The uniformly ^{14}C-ring-labeled TNT (chemical and radiochemical purity >98%; purchased from Prof. Dr. G. Fels, Department of Chemistry, University of Paderborn, Germany) had a specific activity of 2.26 mCi/mmol of TNT and was used at activities of 1823 kBq (= 49.27 μCi and 1729 Bq (= 46.73 μCi in experiments A and B, respectively). Prior to incubation, the additional amounts of TNT and [^{14}C]TNT were sprayed over the soil/molasses mixtures in solutions of methanol (60 % vol). The spraying of TNT solutions was accomplished by rigorously stirring the soil/molasses mixtures to achive an extensive homogeneous distribution of contaminants. After 5 weeks of anaerobic incubation, three bioreactors (one with [^{14}C]TNT, one with normal TNT, and one control) were analyzed, whereas the remaining three reactors (with [^{14}C]TNT, normal TNT, and control) were analyzed after 9 weeks of incubation (5 week of anaerobic plus 4 week of aerobic treatment). All experiments were carried out as unique samples.

Analytical Procedures. Levels of TNT, 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (4ADNT), 2,4-diamino-6-nitrotoluene (24DANT), and 2,6-diamino-4-

nitrotoluene (26DANT) in the extracts were determined by reversed-phase HPLC with a Pharmacia liquid chromatograph equipped with a model 2150 solvent pump, a model 2151 variable-wavelength detector, a model 2157 autosampler, and a model 2152LC controller. The isocratic eluent was methanol:water (35:65 [vol/vol]). The 10-μL aliquots were injected into a Hypersil-ODS (C18) column (25 cm by 4 mm; 5 μm pore size), and the solvent rate was 1 mL/min. The UV detector was set at 254 nm. The efficiency of HPLC analysis was not effected by the relatively high amount of molasses (20 wt %), but the guard column was renewed when the pressure exceeded the upper limit of 350 bar.

The ^{14}C activity was determined using a β-scintillation counter model 1415 from Wallac (Wallac Oy, Turku, Finland) with a System 1400 workstation. The counting time was 1 min, and the counting efficiency (after quench correction) was always above 85%. Some samples (e.g., the dark brown colored humic and fulvic acid fractions) were diluted 1:50 or 1:100 before starting the radioactivity measurement in the liquid scintillation counter (LSC). All determinations in the LSC were performed as triplicate measurements of duplicate samples. For all measurements in LSC, 1 mL of the samples (extracts, Opti-Fluor, and Carbo-Sorb) was mixed with 9 mL of Instant Scintillation Gel (a universal liquid scintillation cocktail for aqueous and nonaqueous samples from Packard Instrument B.V. Chemical Operations). The total ^{14}C in the remaining soil was determined by combustion of three subsamples (1.0, 0.5, and 0.25 g dry weight of the same soil) in a Biological Material Oxidizer model OX-300 from Zinsser Analytic GmbH (Frankfurt, Germany) under the following conditions: Temperature of catalyst, 700 °C; combustion temperature, 900 °C; nitrogen flow, 350 cm^3/min; oxygen flow, 350 cm^3/min; and combustion time cycle, 4 min. The released $^{14}CO_2$ was absorbed in 15 mL of Carbo-Sorb of which 1 mL was analyzed in LSC. For these samples, the counting efficiency was above 92%.

Extraction Procedures. After the anaerobic and anaerobic−aerobic treatment, the soil/ molasses mixtures were dried at 60 °C overnight to remove any moisture. Thereafter, 100 mL of water was added to each reactor vessel and was extracted for 30 min in an ultrasonic bath. Subsequently, the soil/water mixtures were filtered to obtain the water extracts. After another drying step (at 60 °C overnight), 100 mL of methanol was added to each reactor vessel and was again extracted for 30 min by ultrasonification. The soil/methanol mixtures were filtered to obtain the methanol extracts. Prior to the last extraction procedure, the mixtures were dried again (60 °C, 12 h) and then exposed to 100 mL of ethyl acetate. After an extraction procedure of 30 min in an ultrasonic bath, the soil/ethyl acetate mixtures were again filtered to gain the ethyl acetate extracts. These ethyl acetate extracts were evaporated to dryness in a vacuum rotary evaporator, and the residues were dissolved in 10 mL of methanol. As measured in preliminary experiments, the TNT recovery from sandy soil after a single methanol or ethyl acetate extraction (30 min in an ultrasonic bath) was in the range of 94−97% (data not shown). At the end of these three sequential extractions, the soils were dried overnight at 60 °C before alkaline hydrolysis of 25 g of dry matter per bioreactor was performed.

Soil Hydrolysis and Humic Acid Extraction. The solvent-extracted soil/molasses mixtures were subjected to alkaline hydrolysis to obtain a purified humic acid fraction, a fulvic acid and polyphenol fraction, and the humin fraction (the so-called solid soil residue fraction). The humic acid extraction was carried out according to the description of Hsu and Bartha (*17*). Therefore, 25 g of dry matter per reactor was hydrolyzed with 50 mL of NaOH (50% [wt/vol]) by boiling in a reflux unit for 2 h. The hydrolysates were then vacuum-filtered. The solid soil residues (humin fraction) that remained in the filter was dried overnight at 60 °C, and subsamples were oxidized at 900 °C (see Analytical Procedures) to obtain the total ^{14}C activity of this fraction. The dark brown filtrate (which contained the humic acid fraction and the fulvic acid/polyphenol fraction) was then subjected to 50 mL of ethyl actetate and extracted for 30 min in an ultrasonic bath to obtain extractable components from this liquid fraction. The ethyl acetate extract was evaporated to dryness, and the residue was dissolved in 10 mL of methanol prior to determinations by HPLC and (if radiolabeled) LSC.

Thereafter, the dark brown liquid fraction was acidified to pH 1 using concentrated HCl until the humic acid precipitated. The precipitated humic acid was dissolved in 0.5 N NaOH and reprecipitated as described before. The purified humic acid was again dissolved in 0.5 N NaOH, and aliquots were diluted and counted for radioactivity as described above. Subsequently, the remaining (acidic) fulvic acid and polyphenol fractions of the two separating steps of the humic acid extraction were combined and also determined for radioactivity in LSC. Because of the strong quenching effect of the dark brown fractions (both humic and fulvic acid fractions), it was necessary to prepare 1:50 or 1:100 dilutions for LSC analyses. The humic acid- and fulvic acid-bound radioactivity was then compared to that bound to the solid soil residues of the humin fraction. To close the mass balance of the entire original soil mass, the radiolabel of the remaining soil (<25 g dry matter) that was solvent-extracted but not alkaline hydrolyzed was obtained by combustion of subsamples to $^{14}CO_2$. The measured ^{14}C activity was then calculated proportionally to the ^{14}C activities of the different soil fractions that were yielded by the humic acid extraction of the 25-g samples and combined with them.

Results

Radioactivity Distribution in the Organic Extracts of the Soil/Molasses Mixtures. After the anaerobic treatment (after 5 weeks of incubation) and a subsequent aerobic treatment (after 9 weeks of incubation) of the soil/molasses mixtures, three different organic extraction procedures were carried out to obtain the amounts of extractable radioactivity. The results are depicted in Figures 2 and 3. The changes of the experimental setup in experiment B led to a decrease of the total extractable radioactivity from ∼40% after anaerobic treatment to slightly more than 9% after the aerobic treatment (Figure 3). This reflects a decrease of nearly 31% of the total radioactivity and is explained by the immobilization of the radiolabeled contaminants as shown in the following sections. By way of contrast, the decrease of total radioactivity in the extracts of experiment A was only about 9% (from ∼54% after 5 weeks to ∼45% after 9 weeks of incubation; see Figure 2). After the anaerobic treatment, the amount of extractable radiolabel was ∼54% in experiment A and considerably lower in experiment B (with a value of ∼40%). This indicates that the transformation of TNT to more polar metabolites and a subsequent incorporation of these compounds during the anaerobic treatment was more effective in experiment B than it was in experiment A. This suggestion is supported by the higher value of radiolabel of the water-extractable fraction that was first determined in a sequence of three extractions. Therefore, the compression of the soil/molasses mixtures during the anaerobic phase seemed to be a very cheap and effective treatment alternative for further bioremediation processes. In addition, the differences during the aerobic treatment (daily stirring of soils in unclosed reactors versus constantly leading gas through unstirred soil piles in closed reactors) clearly demonstrated the fixation of radiolabel to be considerably higher in experiment B (only 9.4% extractable radioactivity in experiment B vs 44.7% of extractable radiolabel in experiment A). The significant decrease of extractable radiolabel after the aerobic treatment in experiment B is

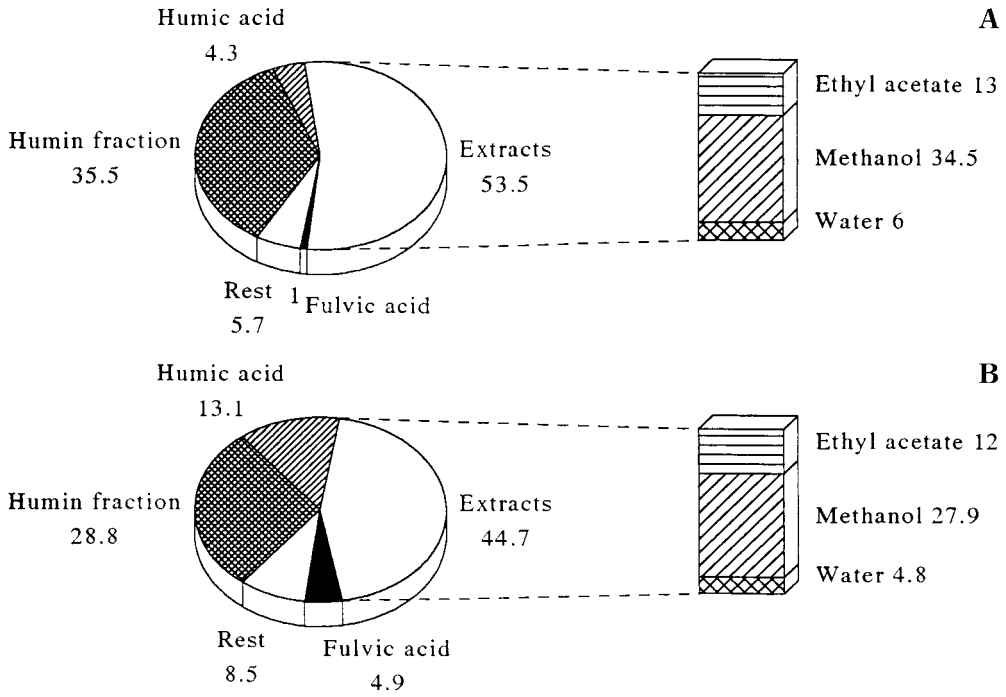

FIGURE 2. Distribution of radioactivity (in %) of the originally applied radioactivity in different soil fractions and in the extracts of the soil/molasses mixtures after anaerobic (5 weeks, A) and anaerobic−aerobic (9 weeks, B) treatment. Results from experiment A. The "rest" fraction contains the values of radiolabel of the ethyl acetate extracts of the hydrolysates, the label of glass and filter materials, and unaccounted label (balance gap). The data are the means of triplicate measurements of duplicate samples of unique bioreactors.

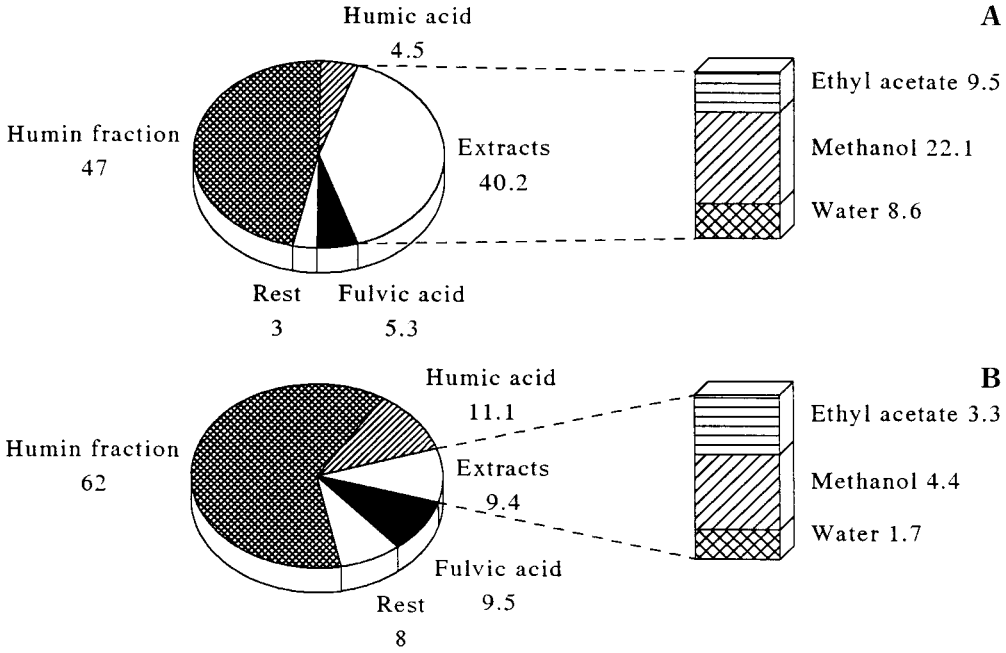

FIGURE 3. Distribution of radioactivity (in %) of the originally applied radioactivity in different soil fractions and in the extracts of the soil/molasses mixtures after anaerobic (5 weeks, A) and anaerobic−aerobic (9 weeks, B) treatment. Results from experiment B. The "rest" fraction contains the values of radiolabel of the ethyl acetate extracts of the hydrolysates, the label of glass and filter materials, and unaccounted label (balance gap). The data are the means of triplicate measurements of duplicate samples of unique bioreactors.

reflected by the amounts of the three extracts that showed losses from ~9% to ~2% in water, from ~22% to ~4% in methanol, and from ~9.5 to ~3% in ethyl acetate extracts (Figure 3). The values of radiolabel in the extracts of methanol and ethyl acetate after the anaerobic treatment of experiment A were very high (~34.5% and ~13%, respectively), which indicated a less effective transformation of TNT because these extracts contained the less to nonpolar metabolites of TNT transformation and parent TNT. These values decreased only slightly after the subsequent aerobic treatment (Figure 2).

Nitroaromatic Contents in the Organic Extracts of the Soil/Molasses Mixtures. The three different extracts of each soil/molasses bioreactor were analyzed by reversed-phase HPLC to obtain the amounts of residual TNT and metabolites produced during microbial cometabolism. The results are summarized in Table 1. Obviously, the levels of radiolabel found in the extracts were not always supported by the levels

TABLE 1. Summary of Concentrations of TNT and Its Reduced Metabolites As Determined by HPLC in the Different Extracts of the Non-Radiolabeled Soil/Molasses Mixtures

	experiment A				experiment B			
	after 5 weeks[a]		after 9 weeks[a]		after 5 weeks		after 9 weeks	
extracts	mg/kg[b]	%[c]	mg/kg	%	mg/kg	%	mg/kg	%
			Water					
TNT	2.5 (± 0.3)	0.4	0	0	0	0	0	0
4ADNT	3.2 (± 0.2)		0		4.9 (± 1.2)		0	
2ADNT	0.8 (± 0.1)		0		0		0	
24DANT	0		0.5 (± 0.1)		0		0	
			Methanol					
TNT	35.0 (± 4.1)	5.1	14.1 (± 2.9)	2.0	42.2 (± 3.6)	6.1	14.5 (± 5)	2.1
4ADNT	50.8 (± 8.2)		40.2 (± 6.6)		195.8 (± 21.4)		15.4 (± 3.8)	
2ADNT	9.5 (± 2.2)		18.9 (± 1.8)		0		0	
26DANT	0		0		9.0 (± 2.0)		0	
24DANT	0		0		18.4 (± 3.4)		3.3 (± 1.1)	
			Ethyl Acetate					
TNT	19.3 (± 3.1)	2.8	17.7 (± 2.5)	2.6	2.0 (± 0.4)	0.3	1.1 (± 0.3)	0.2
4ADNT	17.5 (± 2.8)		2.0 (± 0.3)		4.9 (± 1.4)		0.6 (± 0.1)	
2ADNT	1.2 (± 0.1)		0.8 (± 0.2)		0		0	
26DANT	0		0		0.8 (± 0.2)		0	
TNT recovery	56.8	8.3	31.8	4.6	44.2	6.4	15.6	2.3

[a] 5 week incubation, anaerobic phase; 9 week incubation, anaerobic plus aerobic phase. [b] The data are the means of triplicate measurements of duplicate samples (standard deviations are given in parentheses). [c] TNT recovery in %. the soil/molasses mixtures were originally contaminated with 690 mg of TNT/kg of dry soil (590 mg/kg TNT spiked with 100 mg/kg TNT).

of TNT and aminonitrotoluenes measured in the same extracts. This may be explained by the facts that there are compounds, especially in the chromatograms of the ethyl acetate and methanol extracts, that were not identified by the methods employed and that all three extracts always showed a very broad peak during the first 2 min of HPLC elution, possibly hiding some very polar metabolites of TNT transformation. Summarily, Table 1 clearly indicates that TNT was transformed more effectively with the experimental setup used in experiment B. The TNT transformation rates of experiment A were ~92% after 5 weeks and ~95.5% after 9 weeks of incubation, whereas levels of ~93.5% after 5 weeks and ~98% after 9 weeks of incubation were achieved in experiment B. No parent TNT was detected in the water extracts after the aerated treatment phase, and only small amounts of 24DANT were found at the end of experiment A. The applied ethyl acetate extractions showed small amounts of TNT and 4ADNT (smaller in experiment B), but they were considerably lower in both experiments after the aerobic treatment of the soil/molasses mixtures. The concentrations of diaminonitrotoluenes found in these extracts were negligible. The bulk of nitroaromatics was detected after the methanolic extraction procedure. In these fractions, the levels of original TNT were highest (5% and 6% after 5 weeks in experiments A and B, respectively), but dropped to values of about 2% (in both experiments) after 9 weeks of incubation. The more effective microbial activity for TNT transformation in experiment B is clearly apparent regarding the high value of 4ADNT production and the values of 26DANT and 24DANT production. But also these contaminants decreased considerably during the aerobic treatment of the soil/molasses mixtures. No peaks indicating the presence of known nitro- and/or aminoaromatic compounds were detected in the extracts of the uncontaminated controls. Only the water extract showed a broad peak during the first 2 min of HPLC elution. Tetranitroazoxytoluenes were also never detected in the extracts of all treated soil/molasses mixtures.

Radioactivity Distribution in the Different Soil Fractions of the Soil/Molasses Mixtures. A more rigorous extraction method was applied in order to extract additional ^{14}C-labeled components or metabolites that were still bound to the soil matrix after extraction with organic solvents. A humic acid extraction by alkaline hydrolysis was carried out for this purpose. As shown in Figure 2, the total radiolabel of the different soil components in sum slightly increased by about 6% during the aerobic phase of experiment A. There were some shifts of ^{14}C activity within the different fractions. The humic acid fraction showed an increase of radioactivity from 4.3% to 13.1%, and the fulvic acid and polyphenol fraction increased from 1% to nearly 5% at the end of sequential anaerobic−aerobic treatment. These radiolabel shifts within the different fractions were explained by the decreasing label of the soil-bound residues in the humin fraction (from 36% to 29%), which was determined by soil combustion with subsequent measurement of the evolved $^{14}CO_2$. As opposed to this, the fixation of radiolabeled compounds in experiment B was considerably higher. As can be seen in Figure 3, the ^{14}C activity of the hydrolyzed soil increased by 26% altogether (from ~57% to ~83% at the end of the experiment) with small increases in the humic and fulvic acid fractions but with a large increase in the humin fraction. There were low quantities of ethyl acetate-extractable radiolabel of the hydrolysates in both experiments (A and B). HPLC analyses of these ethyl acetate extracts (dried and dissolved in methanol) allowed traces of diaminonitrotoluenes to be obtained as well as a few unidentified compounds with still aromatic structure as indicated by UV absorption at 254 nm (data not shown). Improvements of this extraction method to identify and measure these compounds in future studies are in progress. As shown in Table 2, the radiolabel in these extracts remained constant during experiment A (0.5−0.6% of total radiolabel initially applied) and only slightly increased during experiment B (from 0.8 to 1.1%). These ethyl acetate extracts clearly showed that small portions of radiolabel (and therefore, of TNT metabolites) were extractable after alkaline hydrolysis, whereas the major part of ^{14}C activity was bound to the humic acid fraction, the fulvic acid fraction, and the humin fraction.

Total Radioactivity Recovery of all Fractions of the Soil/Molasses Mixtures. The most important results are listed in the first three lines of Table 2, because the values of extractable and fixed (incorporated) radiolabel are summed up. The radiolabel values of the different soil components

TABLE 2. Summary of Radioactivity Recovery (in %) of the Originally Applied Radioactivity in the Different Fractions of the Soil/Molasses Mixtures after Anaerobic (5 Weeks) and Anaerobic−Aerobic (9 Weeks) Treatment

	experiment A		experiment B	
	after 5 weeks	after 9 weeks	after 5 weeks	after 9 weeks
extracts[a]	53.5	44.7	40.2	9.4
soil components[b]	40.8	46.8	56.8	82.6
ethyl acetate extracts[c]	0.5	0.6	0.8	1.1
gas phase[d]	0.004	0.035	0.001	ND[f]
glass and filter materials[e]	0.5	0.4	0.4	0.5
total radiolabel recovery	95.3	92.5	98.2	93.6

[a] The values from the corresponding Figures 2 and 3 are combined. [b] The values of the soil components (humin fraction, humic acid fraction, and fulvic acid fraction) from the corresponding Figures 2 and 3 are combined. [c] These are the ethyl acetate extracts from the alkaline hydrolysates. [d] The mean values of absorbed ^{14}C-labeled volatile organic compounds and of $^{14}CO_2$ are combined. The data are the means of triplicate measurements of duplicate samples. [e] The alkaline washing solution of the materials used in the experiments was measured in LSC. The data are the means of triplicate measurements of duplicate samples. [f] ND, not determined.

(humic acid fraction, fulvic acid fraction, and humin fraction) and of the ethyl acetate extracts of the hydrolysates were added (lines 2 and 3 of Table 2), which yielded the following levels of fixed (incorporated) radioactivity: experiment A, 41.3% after 5 weeks and 47.4% after 9 weeks of incubation, which reflects an increase of about 6%; experiment B, 57.6% after 5 weeks and 83.7% after 9 weeks of incubation, which reflects an increase of about 26%. The value obtained after the anaerobic treatment of the soil/molasses mixtures of experiment B was higher than the final value of the total experiment A (57.6% vs 47.4%), and the high final value of experiment B (nearly 84%) was not reached in experiment A. These values clearly underlined the effect that the changes of treatment procedures during anaerobic and aerobic soil remediation had on the incorporation rates of radiolabeled contaminants. Another putative carbon flow pathway to be checked was the degradation of [^{14}C]TNT to radiolabeled organic volatile compounds (by trapping in Opti-Fluor) and/or its mineralization to $^{14}CO_2$ (by trapping in Carbo-Sorb). The ^{14}C activities recoverable in the gas phases were negligible (see Table 2, line 4), so that the loss of radiolabel through the gas phase of the daily stirred soil/molasses reactors (experiment B) was not detected. The reactors were incubated unclosed and were allowed to use the normal air oxygen. The residual radioactivity that remained in the glass and filter materials was also measured. As can be seen in Table 2, the alkaline washing solutions of the cleaned materials at the end of each treatment phase had a radiolabel ranging between 0.4 and 0.5% of the total label originally applied. An overall balance of all radiolabeled components resulted in high recovery values ranging from 92.5% to more than 98% of the radioactivity that was originally applied.

General Observations during the Anaerobic−Aerobic Bioremediation Process. During the anaerobic treatment in experiments A and B, the pH decreased from 6.9−7.0 at the beginning to 4.5−5.0 at the end of incubations. This is explained by the production of acids during the anaerobic microbial metabolism (e.g., fermentations), but the soil/molasses mixtures were not investigated for these compounds. Nevertheless, in other experiments we found lactate and acetate as major intermediate substrates in similar experiments (unpublished data). In the closed reaction vessels, the water content remained constant, but slight losses of solids (approximately 3 g/reactor) were observed during anaerobic treatment. After the aerobic treatment in experiment A, the bioreactor had a water content of only 6 wt %, which additionally may explain the less effective microbial transformation of TNT and metabolites and the less effective incorporation of radiolabeled compounds into the soil matrix as compared to experiment B in which the water content was kept on a constant level (∼30 wt %). At the end of the aerobic treatment in experiment A, white to bluish fungi mycelia and spores were observed, which indicated microbial activity. Identical fungi mycelia and spores were observed during the aerobic bioremediation process of experiment B after 4 days of incubation. In these bioreactors, hydrolytic processes were observed after 16 days, because the production of hydrolytic water moistened the soil/molasses mixtures so that no tap water had to be added. The loss of solids was approximately 7 g/reactor after the aerated incubation phase, which is assumed to result from the complete consumption of sugar (sugar content of molasses slivers was about 22.5 wt %, which corresponded to 2.25 g of sugar/10 g of initially applied molasses slivers) and other components of the soil/molasses mixtures. Total organic carbon measurements were not carried out. All these observations were also made in the uncontaminated controls, so we assume that there was no toxic influence of TNT and metabolites on microbial behavior and activity. On the basis of these obervations, microbial activity was considered to be high. In a previously published study, Bruns-Nagel et al. (*13*) described the activity of the endogenous soil microflora by using the MPN (most probable number) method. Additionally, a pure culture of an aerobic, rod-shaped bacterium capable of transforming TNT was isolated from this soil (unpublished data).

Discussion

The results presented in this study show that a sequential anaerobic−aerobic treatment system stimulated the depletion of TNT by transformation and/or formation of unextractable soil-bound residues by means of integration into the humification process. The latter process might be a significant route of depletion in soil, especially for those xenobiotics that are mineralized only slowly (e.g., explosives, pesticides, polyaromatic hydrocarbons). We assume that the molasses-mediated biogenic binding of radiolabeled TNT metabolites in soil (at least to some extent) is due to processes resembling those described for enzyme-mediated pesticide binding in soils, because the metabolites of TNT with their reactive functional groups (e.g., amino and hydroxylamino groups were identified in degradation studies) (*1, 14, 16, 18, 19*) were predestinated to oxidative soil coupling processes (*20−23*). A report recently published by Dawel et al. (*24*) clearly underlines the importance of reduced functional groups of TNT metabolites for such coupling reactions. The authors proposed the covalent coupling of 24DANT to a laccase-mediated diphenoquinone (resulting from dimerization of two guaiacol monomers and subsequent oxidation of the intermediate) as a model for organic soil matrix components. Interestingly, 4ADNT did not react significantly with these guaiacol condensation products. So the authors concluded that at least two amino groups are necessary for coupling reactions; however, 26DANT and TAT (triaminotoluene) were not tested in their study (*24*). Considering our experiments, we assume that the reduced forms of TNT were easily produced under anoxic conditions by microbial activities (with the nitro substituents serving as additional electron acceptors) (*1, 19*). They yielded relevant amounts of diaminonitrotoluenes (24DANT and 26DANT) that then were coupled to the soil organic matrix by oxidative reactions under the subsequent oxic conditions. Under these circumstances, nearly 84% of the initially applied radiolabel could not be extracted from the soil with usual organic solvent extractions and therefore is to be regarded as strongly bound to the organic soil matrix. However, the recovery of

radiolabeled residues of TNT and metabolites did not allow any statement on the structure of such bound molecules. The measured radioactivity accounts only for single-labeled carbon atoms, whereas it can be stated that there was no significant mineralization of TNT in our experiments (Table 2). In a study by Kaplan and Kaplan (*12*), 22.1% of radioactivity of the initially applied [^{14}C]TNT was bound to the humic fraction in composting experiments, while 35% of radiolabel was not extractable from [^{14}C]TNT-contaminated soil in another study (*25*). Comfort et al. (*26*) demonstrated that 34.6% of radiolabeled TNT was not extractable from solid humic particles of soil slurries after long-term sorption experiments. Another study showed that the bulk of radiolabel accumulated in a nonextractable but hydrolyzable fraction (56.8%), whereas 4.7% of the radiolabel was recovered by combustion of the residue (nonhydrolyzable fraction) (*11*). Regarding the extractable radioactivity and contents of nitroaromatics in the water extracts (simulating wash-out effects) in our experiments, we concluded that the bioremediation procedure used in this study was very effective (no aromatics and only low radiolabel at the end of experiment B). Therefore, it represents an excellent candidate for bioremediation of sites that are contaminated with TNT and perhaps other explosives. The strategy of soil compression during the anaerobic treatment phase and the daily stirring of the mixtures plus keeping the water content on a constant level during the aerobic treatment phase was important for a successful transformation and incorporation of TNT metabolites. Lenke et al. (*15*) also recommended a two-stage bioremediation process of contaminated soils, but they produced a slurry of 18 m^3 of soil and 10 m^3 of water during the anaerobic phase (using sucrose as auxiliary substrate) with subsequent dewatering of the sludge prior to the aerobic treatment. By way of contrast, the compressed soil/molasses mixtures in the present study contained only 30% of water (wt %), so the procedure of soil draining was not necessary prior to the aerobic treatment. We suggest that composting methods rather than the addition of chemicals or pure enzymes should be used as a cheap and effective method of soil bioremediation and detoxification. Compost material stimulates both the turnover of xenobiotical carbon (transformation and/or mineralization) and its long-term binding to the soil organic matrix. TNT metabolites possibly react in similar manner with the soil organic matrix as it was shown for polycyclic aromatic hydrocarbons. Recently, Eschenbach et al. (*27*) and Richnow et al. (*28*) reported on the transformation and (long-term) binding of ^{14}C- and ^{13}C-labeled PAHs to soil. They demonstrated that, for example, anthracene and pyrene and their metabolites were incorporated into the macromolecular organic matter (humic material) during microbial degradation. These authors also emphasize that the formation of nonextractable residues during microbially mediated degradation and naturally occurring humification processes in soils and sediments represents a major sink for organic pollutants. But nevertheless, more long-term studies should be carried out in the future to investigate to what extent xenobiotic hydrocarbon is involved in the natural turnover process of humic substances. Some authors estimated the annual turnover rate of humic bound residues to range between 2% and 8% (*29, 30*). In future work, we will further improve the treatment conditions to obtain higher levels of radioactivity coupling of TNT metabolites to the soil organic matrix. Additionally, we will focus our investigations on the identification of the binding types of TNT metabolites using ^{15}N-labeled TNT and nuclear magnetic resonance (NMR) spectroscopy.

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